sec. These citations are Ahmed and Ivashkiv (2000) J. Immunol. 165:5227-5237, which is cited on page 54, lines 12 and 20-21 of the Specification, and Stancato, et al. (1996) J. Biol. Chem. 271:4134-4137, which is cited on page 54, line 4 of the Specification. See, e.g., legend to Fig. 1 of Ahmed and Ivashkiv (2000) supra; and methods section of Stancato, et al. (1996) supra.

Applicants believe that no new matter is added by way of amendment. Entry of the above amendment is therefore respectfully requested.

Applicants believe that no additional fees are due with this communication. Should this not be the case, the Director is hereby authorized to debit any changes or refund any overpayments to DNAX Deposit Account No. 04-1239. If the Examiner believes that a telephone conference would aid the prosecution of this case in any way, please call the undersigned.

Respectfully submitted,

Date: July 10, 2002

By: _____
Sheela Mohan-Peterson
Attorney for Applicants
Reg. No. 41,201

DNAX Research, Inc.
901 California Avenue
Palo Alto, California 94304-1104
Tel No.: (650) 496-6400
Fax: (650) 496-1200

Enclosures:

(1) Ahmed and Ivashkiv (2000) J. Immunol. 165:5227-5237.

(2) Stancato, et al. (1996) J. Biol. Chem. 271:4134-4137.

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the specification:

The paragraph on page 74, lines 22-27, has been amended as follows:

Table 1 below illustrates the results of western blot analysis. AK155 treatment for five [seconds] minutes was sufficient for induction of detectable levels of STAT3 phosphorylation. A lysate of the herpes virus samari-transformed T-cell line CB-15 served as a positive control (Table 1). Herpes virus samurai transformed T-cells secrete large amounts of AK155 (Knappe *et al.* (2000) J. Virol. 74:3881-3887) and constitutively phosphorylate STAT3.

The paragraph on page 75, lines 1-4, has been amended as follows:

Table 1. Colo-205 cells treated with HIS-AK155 (10 ng/ml final concentration).

Table 1.	0 sec	5 [sec] min	10 [sec] min	20 [sec] min	30 [sec] min	60 [sec] min	CB-15 lysate
Phosphorylated STAT3	---	+++	+++	++++	++++	++++	++

Inhibition of IL-6 and IL-10 Signaling and Stat Activation by Inflammatory and Stress Pathways[1]

Simi T. Ahmed* and Lionel B. Ivashkiv[2]*†

The development and resolution of an inflammatory process are regulated by a complex interplay among cytokines that have pro- and anti-inflammatory effects. Effective and sustained action of a proinflammatory cytokine depends on synergy with other inflammatory cytokines and antagonism of opposing cytokines that are often highly expressed at inflammatory sites. We analyzed the effects of the inflammatory and stress agents, IL-1, TNF-α, LPS, sorbitol, and H_2O_2, on signaling by IL-6 and IL-10, pleiotropic cytokines that activate the Jak-Stat signaling pathway and have both pro- and anti-inflammatory actions. IL-1, TNF-α, and LPS blocked the activation of Stat DNA binding and tyrosine phosphorylation by IL-6 and IL-10, but not by IFN-γ, in primary macrophages. Inhibition of Stat activation correlated with inhibition of expression of IL-6-inducible genes. The inhibition was rapid and independent of de novo gene induction and occurred when the expression of suppressor of cytokine synthesis-3 was blocked. Inhibition of IL-6 signaling was mediated by the p38 subfamily of stress-activated protein kinases. Jak1 was inhibited at the level of tyrosine phosphorylation, indicating that inhibition occurred at least in part upstream of Stats in the Jak-Stat pathway. Experiments using Stat3 mutated at serine 727 and using truncated IL-6Rs suggested that the target of inhibition is contained within the membrane-proximal region of the cytoplasmic domain of the gp130 subunit of the IL-6 receptor and is different from the SH2 domain-containing protein-tyrosine phosphatase/suppressor of cytokine synthesis-3 docking site. These results identify a new level at which IL-1 and TNF-α modulate signaling by pleiotropic cytokines such as IL-6 and IL-10 and provide a molecular basis for the previously described antagonism of certain IL-6 actions by IL-1. *The Journal of Immunology,* **2000, 165: 5227–5237.**

Interleukin-6 is a multifunctional cytokine that plays an important role in immune and inflammatory responses (reviewed in Ref. 1). IL-6 works by regulating the expression of immune/inflammatory genes and regulating cell proliferation, differentiation, and survival. Since IL-6 expression is elevated in inflammatory diseases and is induced by inflammatory stimuli, such as IL-1 and TNF-α, IL-6 has been considered a proinflammatory cytokine (1–3). Many of its proinflammatory and immune properties are secondary to potent effects on driving B cell Ab production, promoting T cell function, and promoting the expression of chemokines and adhesion molecules on endothelial cells (1, 4, 5). In contrast to its effects on lymphocytes and endothelial cells, IL-6 and the related cytokine IL-11 that signals through the same receptor subunit (see below) have suppressive effects on macrophages (6, 7), astrocytes (8), and fibroblasts (9) and suppress the expression of IL-12, IFN-γ, TNF-α, adhesion molecules, and proteases both in vitro and in vivo (6–12). In IL-6 knockout mice, there is decreased IL-10 production and increased IL-12 production from macrophages compared with those in normal mice (12). One mechanism that underlies the anti-inflammatory effects of IL-6 and IL-11 appears to be inhibition of NF-κB (13). IL-6 also induces the expression of multiple factors with anti-inflammatory properties, including IL-1R antagonist, soluble TNF receptors, IL-10, acute phase reactants, glucocorticoids, protease inhibitors (such as tissue inhibitor of metalloproteinase-1), and suppressors of cytokine signaling (SOCS)[3] proteins (12, 14–20). Consistent with these anti-inflammatory effects, IL-6 has been shown to attenuate inflammatory lung disease (21) and to play a chondroprotective role in zymosan-induced arthritis (22), and IL-11 is an effective anti-inflammatory agent in collagen-induced arthritis (23) and psoriasis (24). These observations suggest that induction of IL-6 and IL-11 expression during inflammation, similar to induction of IL-10, may contribute to a negative feedback loop. The overall roles of IL-6 and IL-11 in a particular inflammatory process are determined by the balance between their pro- and anti-inflammatory actions on different cell types.

IL-6 is one member of a family of related cytokines (IL-6, IL-11, oncostatin M (OsM), leukemia-inhibitory factor (LIF), and cardiotropin) that bind to receptors that consist of cytokine-specific α-chains and a shared gp130 receptor subunit that functions in signal transduction (1). The α subunits determine the specificity of binding of these cytokines to their receptors and in the case of LIF and OsM also contribute to signaling. The α subunits specific for IL-6 and IL-11 play no known role in signal transduction, and thus signals generated by IL-6 and IL-11 are similar, since in both cases they are mediated by gp130 homodimers. Some of the differences in biological activity of IL-6 and related cytokines are probably explained by cell type-specific expression of receptor α subunits.

*Graduate Program in Immunology, Weill Graduate School of Medical Sciences, and †Department of Medicine, Hospital for Special Surgery, Weill Medical College of Cornell University, New York, NY 10021

[illegible]

[illegible]

[illegible]

[illegible]

Tyk2 and subsequent tyrosine phosphorylation and activation of latent cytoplasmic Stat transcription factors (25). In many cells, including myeloid cells, IL-6 activates predominantly Stat3, although Stat1 can be activated by high doses of cytokine in certain cell types. Stat3 has different, and even opposite, functions in different cells depending upon cell type and activation status, can induce either proliferation or growth arrest, and may have both pro- and anti-apoptotic properties. In the immune system, Stat3 promotes T cell survival and function (26) and B cell Ab production (27). In contrast, in myeloid cells, deletion of Stat3 results in hyperactivation of macrophages, dramatic increases in inflammatory cytokine production, and inflammatory bowel disease, thus indicating a role for Stat3 in down-regulation of macrophage activation (28).

IL-1 and TNF are major inflammatory cytokines that activate the expression of adhesion molecules, chemokines, and cytokines (2, 3). IL-1 and TNF activate several signaling pathways, leading to the downstream activation of NF-κB transcription factors and activation of c-Jun N-terminal kinases (JNKs) and the p38 kinases (29), collectively termed stress-activated protein kinases (SAPKs), that constitute two subfamilies of mitogen-activated protein kinases (MAPKs) (30). SAPKs are also activated by cellular stressors, including reactive oxygen intermediates, osmolar shock, and UV radiation, some of which are present during inflammation. SAPKs phosphorylate and activate transcription factors, including AP-1, and have been strongly implicated in mediating IL-1 and TNF inflammatory effects (29, 30). IL-1 and TNF are expressed at most inflammatory sites, where they regulate the expression of other cytokines and interact with other immune/inflammatory cytokines in a cytokine network (31). Interestingly, both synergistic and antagonistic interactions of IL-1 and TNF with cytokines of the IL-6 family have been described. IL-1 and IL-6 work together in the induction of type I acute phase protein genes (14), metalloproteases (32), and HIV expression (33). In contrast, IL-6, IL-11, and OsM antagonize IL-1-, TNF-, and LPS-induced expression of tissue inhibitor of metalloproteinase-3 (9), adhesion molecules (8), TNF-α, IL-8, and GM-CSF (6, 10, 24) and inhibit IL-1-dependent cartilage degradation (34), possibly by a mechanism that involves inhibition of NF-κB (13). Going in the other direction, IL-1 and TNF block IL-6 induction of type II acute phase response genes such as thiostatin and fibrinogen (14, 35) and inhibit IL-6-induced proliferation of thymocytes (36). The mechanisms underlying the antagonism of the actions of IL-6 by IL-1 or TNF are not known.

We have previously described a novel and rapid mechanism of inhibition of IL-6 signaling that was mediated by the extracellular stimulus-regulated kinase (ERK) subfamily of MAPKs (37, 38). Within minutes of activation, ERKs were found to inhibit IL-6-mediated activation of Stat3 via a mechanism that probably involved modification of existing signaling components in the cell. Importantly, this inhibition was found to occur upstream of Stat3 in the Jak-Stat cascade, such that Stat3 did not become tyrosine phosphorylated. Given that ERKs and SAPKs can work together by phosphorylating similar motifs on common or different sub- [illegible]

[illegible] be accomplished by the p38 subgroup of the SAPKs. This inhibitory pathway is activated by stimuli different from those that activate ERK-dependent inhibition of IL-6 and appears to have a [illegible]

Materials and Methods

Cell isolation and tissue culture

Monocytes were obtained from PBMC immediately after isolation or after 2 days of culture using a kit to deplete nonmonocytic cells (Miltenyi Biotec, Auburn, CA) or by positive selection using anti-CD14 magnetic beads as recommended by the manufacturer (Miltenyi Biotec) and were >97% pure as verified using flow cytometry as previously described (39). Monocytes were used fresh or after 2 days of culture under adherent conditions under which differentiation into macrophages was initiated, as assessed by increased cell size and granularity. Similar results were obtained regardless of the method of monocyte purification or culture. MM6 human myeloid cells (40) and U266 human myeloma cells were cultured in RPMI, and 293T cells were cultured in DMEM supplemented with 10% FBS. When agents that were dissolved in DMSO were used (PD98059, SB203580), DMSO was added to control cells to keep concentrations of DMSO (0.1 or 0.2%) equal in all wells.

EMSAs

Cell extracts were prepared as previously described (41). Extracts corresponding to 3.3×10^5 cells (~ 12 μg of protein) were incubated for 15 min at room temperature with 0.5 ng of ^{32}P-labeled double-stranded high-affinity SIS-inducible element (hSIE) oligonucleotide (38) in a 15-μl binding reaction containing 40 mM NaCl and 2 μg of poly(dI-dC) (Pharmacia, Piscataway, NJ), as previously described (41), and complexes were resolved on nondenaturing 4.5% polyacrylamide gels.

Immunoblotting, immunoprecipitation (IP), and kinase assays

Cell lysates or immunoprecipitates (see below) were fractionated on 7.5% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and incubated with phospho-specific (Tyr^{705}) Stat3 Ab, phospho-specific (Thr^{180}/Tyr^{182}) p38 Ab, phospho-specific (Thr^{202}/Tyr^{204}) ERK1/2 Ab (New England Biolabs, Beverly, MA), monoclonal Stat3 and ERK1/2 Abs (Transduction Laboratories, Lexington, KY), anti-phosphotyrosine Ab (4G10, Upstate Biotechnology, Lake Placid, NY), FLAG Ab (M2), and p38, Jak1, and JNK1 Abs (Santa Cruz Biotechnologies, Santa Cruz, CA). For immunoprecipitations, extracts corresponding to $10-20 \times 10^6$ cells were adjusted to a 0.5-ml volume in IP buffer (38) and incubated with 4 μg of FLAG, Stat3, Jak1, or JNK1 Abs (Santa Cruz Biotechnologies). Immunoprecipitates were collected using protein G- and protein A-agarose beads and washed three times with IP buffer, and once with PBS. For kinase assays, 25% of the IPs were saved for immunoblot analysis, and the remaining 75% were washed and resuspended in 50 μl of kinase buffer. JNK kinase activity was assayed by incubation at room temperature for 30 min with 10 μCi of [γ-^{32}P]ATP and 5 μg of GST-Jun substrate.

Nuclear extract preparation

A nuclear miniextract procedure (42) was used with modifications. MM6 cells (8×10^6) were washed in HBSS, resuspended, and incubated in 160 μl of buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, and 1 mM Pefablock SC (Roche, Indianapolis, IN)) for 7 min on ice followed by a 2-min incubation with Nonidet P-40 (final concentration, 0.2%). The nuclear fraction was pelleted by centrifugation and lysed in buffer containing 20 mM HEPES (pH 7.0), 300 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 0.1% Triton X-100, 0.5 mM DTT, 200 μM PMSF, and 20% glycerol. The protein concentration of the extracts was determined using the Bradford assay.

Analysis of mRNA levels

Total cellular RNA was isolated using TRIzol (Life Technologies, Gaithersburg, MD) according to the instructions of the manufacturer. For RT-PCR, RNA was treated with RNase-free DNase, and cDNA was obtained using Moloney murine leukemia virus reverse transcriptase (Life Technol- [illegible]

[illegible] P]dATP was added to each reaction. No amplification products were obtained when reverse transcriptase was omitted, indicating the absence of contaminating genomic DNA. Amplification was empirically determined to be in the linear range. Oligonucleotide primers used are [illegible]

GTG CTG AGT TGG CAG and GCT TTC AAT CCA AAG CCA GAA; and protein that interacts with activated Stat (PIAS3), GCC CAC CAC CCT TGC ATC and GCT CGG CCC ATT CTT GGT. PGE_2 receptor primers were provided by C. Beadling and K. Smith (44).

Transient transfections and reporter gene assays

293T cells were transfected in duplicate in 100-mm dishes, using the calcium phosphate coprecipitation technique with expression plasmids encoding constitutively active (CA-)MAPK kinase 3 (MKK3) and CA-MKK6 (provided by R. Davis (45)), a CA-MEK1 encoding plasmid (containing the S218E and S222D mutations and an amino-terminal deletion of residues 30–49), or a control empty vector. Cells were cotransfected with plasmids encoding carboxyl-terminal FLAG-tagged Stat3 (46), and β-galactosidase, and the total amount of DNA added per transfection was 20 μg. Six hours post-transfection, cells were washed and cultured with fresh medium for another 18 h, split onto replicate 60-mm tissue culture dishes, allowed to incubate for an additional 24 h, and stimulated with cytokines. Transfection efficiency was monitored by assaying for β-galactosidase activity. In reporter gene experiments, a plasmid encoding 4X-IRF-γ-activated sequence-luciferase (46) was used, and luciferase activity was normalized for β-galactosidase activity encoded by a cotransfected internal control plasmid. Each transfection experiment was performed three to five times.

Results

IL-1, TNF-α, LPS, and stress agents inhibit activation of Stats by IL-6 and IL-10, but not by IFN-γ, in primary macrophages and myeloid and B cell lines

The effects of inflammatory cytokines and LPS on IL-6 signaling and Stat activation were determined using primary monocytes/macrophages. IL-6 treatment of monocytes resulted in the rapid induction of DNA-protein complexes that bound the hSIE oligonucleotide (Fig. 1*A*, *top panel*, *lane 2*), which, as previously reported (41, 47), contained Stat1 and Stat3, based on supershift assays with specific Abs (data not shown). A 20-min pretreatment with TNF-α, IL-1, or LPS inhibited Stat DNA binding (*lanes 3–5*). Tyrosine phosphorylation of Stat1 and Stat3 at a conserved tyrosine residue is necessary for dimerization and DNA binding (25). IL-6 induced tyrosine phosphorylation of both Stat1 and Stat3 was inhibited by TNF-α, IL-1, and LPS (Fig. 1*A*, *second* and *fourth panels*) in a manner that correlated with inhibition of DNA binding (Fig. 1*A*, *top panel*). Immunoblotting of the same extracts showed comparable levels of Stat1 and Stat3 proteins in all lanes (Fig. 1*A*, *panels 3* and *5*), demonstrating that the observed inhibition of DNA binding and tyrosine phosphorylation was not secondary to reduced Stat protein levels. Since IL-6 may have both pro- and anti-inflammatory effects on macrophages, the effects of inflammatory stimuli on signaling by IL-10, a potent inhibitor of macrophage function (48), were determined. As previously reported (49), IL-10 activated predominantly Stat3 in macrophages (Fig. 1*B* and data not shown), and similar to IL-6, IL-10 activation of Stat3 was inhibited by TNF-α, IL-1, and LPS at both DNA binding and tyrosine phosphorylation levels (Fig. 1*B*). In contrast, activation of Stat1 by the proinflammatory cytokine IFN-γ was not inhibited (Fig. 1*C*). These results are distinct from those reported by Stoiber et al. (50), who showed that long periods of preincubation with LPS (4–48 h) suppressed IFN-γ signaling. These results, therefore, suggest that at early time points after addition to cells, inflammatory cytokines preferentially block Jak-Stat signaling by cytokines that have anti-inflammatory properties. Since Stat3 clearly subserves an anti-inflammatory function in myeloid cells (28), inhibition of Stat3 activation suggests that IL-1, TNF-α, and LPS block anti-inflammatory signals generated by IL-6 and IL-10.

To assess whether inhibition of Jak-Stat signaling by inflammatory factors occurs in other cell types and to identify cell lines that would be useful for further mechanistic studies, the effects of inflammatory and stress factors on Stat activation were assessed in MM6 myeloid cells, U266 myeloma cells, 293T cells, primary fibroblasts, and HepG2 cells. Because not all these cells express significant levels of IL-1Rs, and different inflammatory cytokines and stress factors activate SAPKs in a cell type-specific manner (30), the stress factors sorbitol (hyperosmolar shock), UV radiation, anisomycin, and H_2O_2 (oxidative stress) were also used.

IL-6 activated primarily Stat3 in MM6 cells (Fig. 2*A*, *top panel*, *lane 2*, and data not shown), and a 20-min pretreatment with IL-1, H_2O_2, UV light, or sorbitol served to inhibit DNA binding by Stat3 (Fig. 2*A*, *lanes 3*, *5*, *6*, and *8*), whereas anisomycin had no such effect. IL-1 did not inhibit IFN-γ-induced Stat1 activation or IL-4-induced Stat6 activation in MM6 cells, demonstrating specificity of inhibition (data not shown). A similar pattern of inhibition of DNA binding was detected with U266 cells (Fig. 2*B*). Inhibition of Stat activation was also observed in 293T cells (with UV, IL-1, sorbitol, and H_2O_2) and primary fibroblasts (only IL-1 tested; data not shown). In HepG2 cells, which are a major target of IL-6 action, IL-1 strongly inhibited Stat3 (Fig. 2*C*). Pretreatment of MM6 and U266 cells with stress agents inhibited accumulation of tyrosine-phosphorylated Stat3 (Fig. 2*D*) in a manner that correlated with inhibition of DNA binding (Fig. 2, *A* and *B*). Our results with myeloid cells agree with those of Bode et al. (51), although our observation with HepG2 cells is in apparent contrast with their results, where they describe weak or no inhibition of IL-6 signaling by, respectively, TNF-α and LPS. This difference may be secondary to inherent differences between IL-1 and LPS/TNF or due to

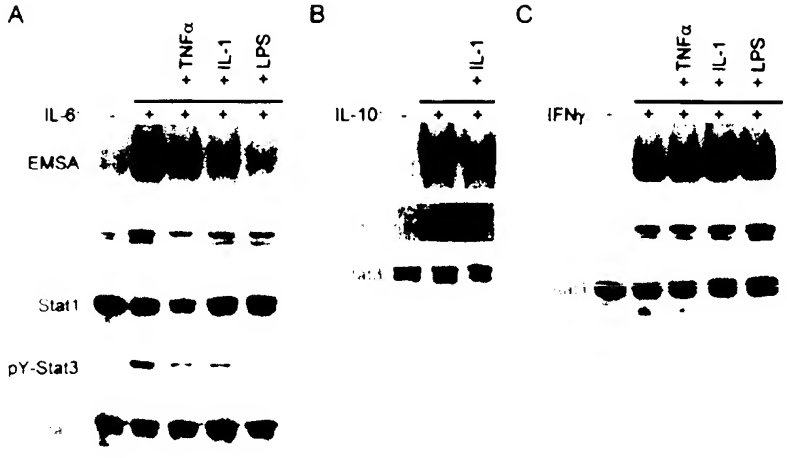


FIGURE 1. Inflammatory cytokines suppress Stat activation by IL-6 and IL-10, but not by IFN-γ, in primary macrophages. Purified macro[illegible]d using magnetic beads, as described in *M[illegible] ethods*, after 2 days of culture were treated fo[illegible]n with TNF-α (100 ng/ml), IL-1β (100 ng/ml), or LI[illegible] μg/ml), followed by [illegible] with IL-6, 50 ng/ml; IL-10 (100

[illegible] with Abs against tyrosine phosphorylated Stat1 or Stat3, followed by probing the same filter with Abs against Stat1 or Stat3. *A*, Two different gels were run to separately analyze Stat1 and Stat3. Similar results

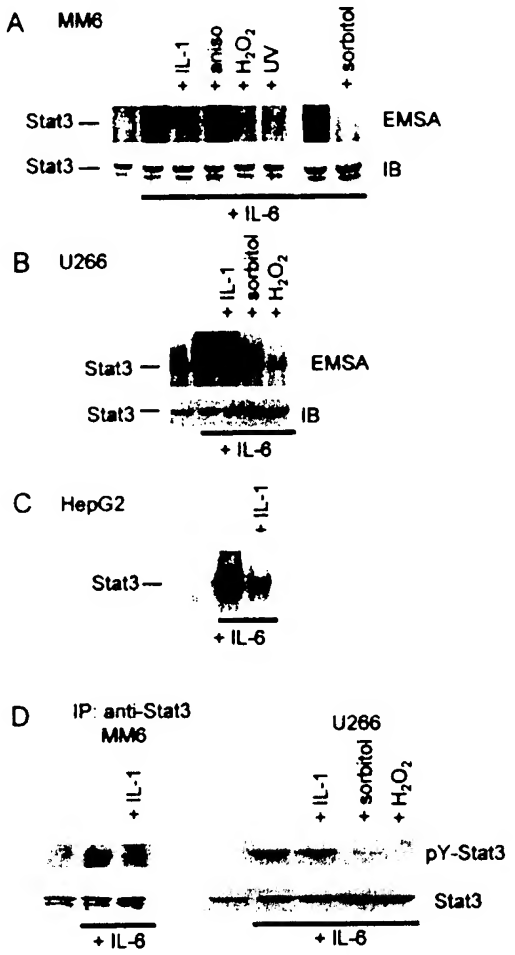


FIGURE 2. Inhibition of IL-6 Stat activation by multiple factors that activate stress kinases. *A*, MM6 cells were treated for 20 min with IL-1 (50 ng/ml), anisomycin (10 μg/ml), H_2O_2 (1.1 mM), UV light (50 J/m^2), or sorbitol (400 mM), followed by IL-6 (50 ng/ml) stimulation (12 min). Whole-cell extracts were assayed for binding to the hSIE oligonucleotide using EMSA (*top*), and the same extracts were analyzed by immunoblotting (IB) with specific Stat3 antiserum (*bottom*). *B*, U266 myeloma cells were used. *C*, HepG2 cells were used. *D*, Stat3 immunoprecipitates (MM6) or whole-cell extracts (U266) were analyzed by immunoblotting with Abs against tyrosine-phosphorylated Stat3 (*top panel*), and the same filter was probed with anti-Stat3 (*bottom panel*). pY, Phosphotyrosine.

inefficient signaling by the latter agents in HepG2 cells. The authors, in fact, indicate that LPS may not be signaling in their system (51). The cell lines tested did not have detectable Stat activation in response to IL-10, and thus the effects of stress factors on IL-10 signaling could not be assessed. Overall, these results indicate that inflammatory and stress factors that activate SAPKs are capable of inhibiting IL-6-triggered Stat activation in several pri-

and Stat3 translocation was analyzed using immunoblotting. Treatment of cells with increasing doses of IL-6 (2.5–50 ng/ml) resulted in proportionately higher levels of Stat3 protein in the nucleus

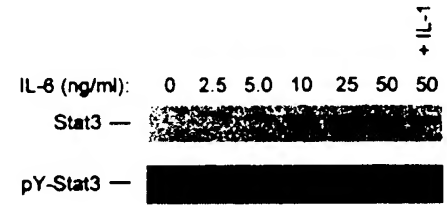


FIGURE 3. IL-1 prevents nuclear translocation of Stat3. MM6 cells were treated with increasing doses of IL-6, and nuclear extracts were assayed for both tyrosine-phosphorylated and total Stat3 protein levels by immunoblotting.

bottom panel). These results show that after IL-1 treatment, Stat3 is prevented from accumulating in the nucleus, where it activates transcription.

Stress agents inhibit expression of IL-6-inducible genes

To assess the functional consequences of inhibition of IL-6 activation of Stats, semiquantitative RT-PCR was used as previously described (39, 43) to determine the effect of inflammatory/stress agents on steady state mRNA levels of IL-6-inducible genes. IL-6 activation of PIAS1, PIAS3 (52), and PGE_2 receptor (44) genes was suppressed by IL-1 (Fig. 4*A*). More than 30 genes were tested, and several patterns of gene regulation were observed, including activation of genes by both IL-1 and IL-6 and inhibition of IL-1 by IL-6 (data not shown). Thus, IL-1 did not cause a generic reduction of IL-6-dependent gene regulation. In U266 cells, IL-6-induced expression of SOCS3, IRF-1, Stat1, and PIAS3 was suppressed by a 20-min pretreatment with sorbitol or H_2O_2 (Fig. 4*B*). Transcription of genes encoding Stat1, IRF-1, and SOCS3 is Stat dependent (19,

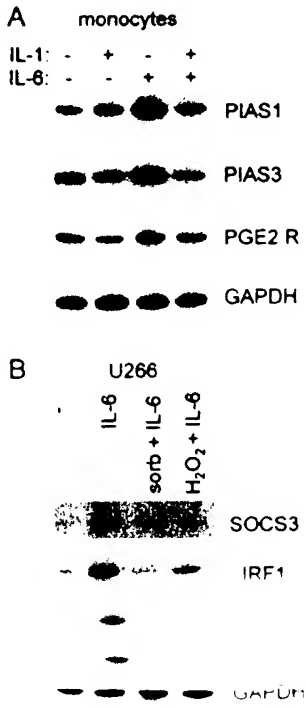
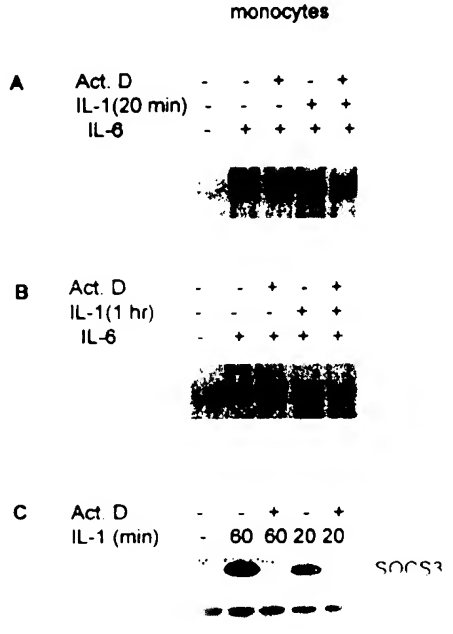


FIGURE 4. Inflammatory stress agents suppress the expression of IL-6-inducible genes. *A*, Monocytes were cultured for 2 days and were treated with IL-1 (200 ng/ml) for 20 min and with IL-6 (50 ng/ml) for 3 h. *B*, U266

25), and thus inhibition of Stat activation probably contributes to inhibition. Interestingly, sorbitol and H_2O_2 did not induce SOCS3 expression (Fig. 4*B*), suggesting that inhibition of signaling in B cells was not mediated by SOCS3 (see below). GAPDH levels were comparable in all lanes, demonstrating that inhibition was not secondary to nonspecific effects of the stress agents on transcription. Thus, inhibition of IL-6 signaling by IL-1 and stress agents bears physiologic consequences with regard to gene expression.

Inhibition of IL-6 signaling by IL-1 is independent of de novo gene expression

Several reports indicate that cytokine signaling can be inhibited by the induction of inhibitory molecules such as SOCS (18–20, 53, 54). Inflammatory cytokines such as TNF induce SOCS3 expression, probably mediated by the p38 pathway, and induction of SOCS3 correlated with inhibition of Stat3 activation in response to IL-6 (51). More recently, association between SOCS3 and gp130 (the signaling subunit of the IL-6R) has been shown to suppress IL-6 signaling (55, 56). We therefore investigated whether the inhibition of IL-6 signaling seen in our system was dependent on de novo gene induction, especially that of SOCS3. Preincubation of primary macrophages with actinomycin D (an inhibitor of RNA synthesis) had no effect on IL-1-mediated inhibition of IL-6 signaling when IL-1 was added either 20 min or 1 h before IL-6 (Fig. 5, *A* and *B*). In addition, although IL-1 induced SOCS3 expression at both time points (Fig. 5*C*, *lanes 2* and *4*), actinomycin D essentially blocked completely this induction (*lanes 3* and *5*). Studies using cycloheximide, a protein synthesis inhibitor, did not yield informative data, as cycloheximide treatment alone significantly blocked IL-6 signaling (data not shown). These results indicate that inhibition of Stat3 activation by IL-6 in our system occurs via a rapid and inducible pathway that does not rely on the expression of new genes.



with IL-1 (50 ng/ml) for 20 min, followed by a 15-min stimulation with IL-6 (50 ng/ml). *B*, The same assay as that in *A* was conducted, except that IL-1 was added 1 h before adding IL-6. *C*, Macrophages in wells parallel

DNA binding	+	+	+	+
ERK kinase	ND			ND
JNK kinase			+	+
p38 kinase	+	+	+	+

Role for p38 in inhibition of IL-6 signaling

Different inflammatory/stress agents activate distinct MAPKs in different cell types (30), and we investigated possible roles played by different MAPKs in inhibition of IL-6 signaling. Activation of ERKs, JNKs, and p38 in MM6 cells by inflammatory/stress factors was determined using both phosphotyrosine/threonine immunoblotting and kinase assays, and the results are summarized in Table I. In addition, in HepG2 cells, IL-1 and other stress agents activated both ERKs and p38 (data not shown). As indicated in Table I, none of the three agents tested activated ERK1/2 over background levels, and only UV and sorbitol activated JNK. The p38 kinase, however, was activated by all agents that also inhibited Stat3 DNA binding, namely, IL-1, H_2O_2, UV, and sorbitol. Interestingly, anisomycin was the only stress agent that did not activate p38 (or the other kinases) in our system, which correlates with its inability to inhibit Stat3 DNA binding (Fig. 2*A* and Table I). These results suggested that p38 may play a role in the suppression of Stat3 activation.

To investigate whether p38 is important in mediating inhibition of IL-6 signaling, SB203580, a specific inhibitor of p38 activation and kinase activity (45), was used. When MM6 cells were incubated with SB203580 before IL-1 treatment, inhibition of Stat3 DNA binding by IL-1 was completely reversed (Fig. 6*A*, *top panel*, *lanes 4* and *5*). Reversal of this inhibition correlated with inhibition of p38 activation (Fig. 6*A*, *middle panel*), suggesting a role for p38 in this process. We have previously shown that PMA treatment blocked Stat3 activation in a fashion that was dependent on the MEK-ERK pathway, which is distinct from the p38 activation pathway, and was reversed by the MEK inhibitor, PD98059 (37, 38). We determined whether ERKs and p38 could play distinct roles in inhibition by different factors. As expected, PMA treatment of MM6 cells resulted in inhibition of Stat3 that was reversed by PD98059 (Fig. 6*B*, *lanes 3* and *4*) and not by SB203580 (*lane 5*). In contrast, IL-1-mediated inhibition was reversed by SB203580 (*lanes 6* and *8*), but not by PD98059 (*lane 7*). This differential sensitivity to kinase inhibitors suggests that different MAPKs can inhibit IL-6 signaling, and that inhibition can be achieved through the activation of distinct signaling pathways.

The role of the p38 stress kinase pathway in inhibition of IL-6 signaling was directly tested using the expression of CA-MKK3 and CA-MKK6 kinases, which are immediately upstream of p38 and activate p38 by phosphorylation (30). This approach has been used extensively, and expression of CA-MKK3 and CA-MKK6 does not result in cross-activation of JNKs or ERKs (45). 293T cells were transfected with either a control empty vector or the same vector encoding the CA-MKK3 and CA-MKK6 kinases and with wild-type Stat3 containing a carboxyl-terminal FLAG tag. CA-MKK3 and CA-MKK6 dramatically suppressed tyrosine phosphorylation of Stat3 in response to IL-6 (Fig. 7, *top panel*,

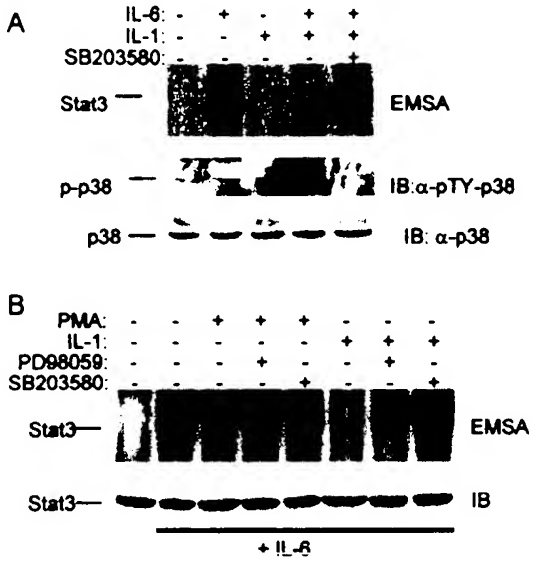


FIGURE 6. Inhibition of IL-6 activation of Stat3 by IL-1 is reversed by the p38 kinase inhibitor SB203580. MM6 cells were incubated with IL-1 (50 ng/ml) for 20 min followed by a 12-min stimulation with IL-6. Cells were preincubated with SB203580 (10 μM) or PD98059 (20 μM) for 45 min. Cell extracts were assayed for binding to the hSIE oligonucleotide using EMSA (*upper panels*). The same extracts were analyzed by immunoblotting (IB) with Abs against activated p38, total p38, or Stat3.

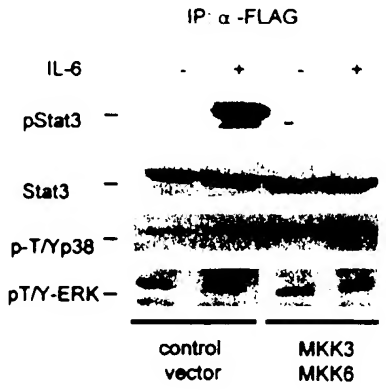


FIGURE 7. Constitutively active MKK3 and MKK6 kinases block Stat3 tyrosine phosphorylation in response to IL-6. 293T cells were cotransfected with a *lacZ* internal control plasmid, FLAG-Stat3, and a control empty vector or the same vector encoding constitutive active MKK3 and MKK6 kinases. After 24 h cells were split onto replicate plates and subsequently stimulated with IL-6 (50 ng/ml) for 12 min. Anti-FLAG-Stat3 immunoprecipitates or cell extracts were analyzed using immunoblotting. Transfection efficiency was comparable, as assessed by measuring β-galactosidase activity.

lane 4). Immunoblotting of the same filter with Abs against the FLAG epitope confirmed equivalence of transfected Stat3 levels in the immunoprecipitates (Fig. 7, *second panel*). Activation of p38 by CA-MKK3 and CA-MKK6 was confirmed (Fig. 7, *third panel*, *lanes 3* and *4*), and activation of ERKs or JNKs was not detected (Fig. 7, *bottom panel*, and data not shown). These results demonstrate that selective activation of the MKK3/6→p38 pathway is sufficient for inhibiting IL-6-triggered Stat3 activation.

To further strengthen the role of the MKK3/6→p38 pathway in regulating Stat3 activation and to assess the functional consequences of this process on the regulation of transcription by Stat3, reporter gene assays were performed using a 4× IRF-γ-activated sequence-luciferase reporter construct that contains four Stat3 binding sites upstream of the thymidine kinase promoter and is dependent on Stats for cytokine-activated transcription (46). Cells were cotransfected with either a control plasmid, CA-MKK3- and CA-MKK6-encoding plasmids, or a CA-MEK1-encoding plasmid (which specifically activates the ERKs). IL-6 treatment resulted in the induction of reporter gene activity, which was strongly inhibited by the CA-MKK3 and CA-MKK6 kinases (Fig. 8). A representative experiment of three performed is shown. Similar to our previous results in HepG2 cells (37), CA-MEK1 also inhibited gene transcription, although inhibition was not as strong as that seen with MKKs (Fig. 8). Weaker inhibition of IL-6-induced reporter gene activity by CA-MEK1 in 293T cells (Fig. 8) relative to

by ERKs (57) and confirms that CA-MEK1 is indeed active in our system. The inhibitory effect of CA-MEK1 or CA-MKK3 and CA-MKK6 was not detected when IFN-γ was used, suggesting that these kinases do not nonspecifically block all Jak-Stat signaling, consistent with the lack of inhibition of IFN-γ Stat1 activation (Fig. 1*C*). These results clearly demonstrate that the MKK3/6 → p38 pathway is capable of inhibiting Stat3-dependent reporter gene activity. They also suggest that this effect may be distinct from inhibition by the MEK→ERK pathway.

Inhibition of IL-6 signaling by MKKs/p38 occurs upstream of Stat3 activation

We have previously shown that inhibition of IL-6 signaling by the ERKs occurs upstream of Stat3 activation (37). Alternatively, others have shown that MAPKs can directly modify Stat3 by phosphorylating it on serine 727 (58–61). We therefore set out to determine which of these mechanisms, if any, were operative in our system. First, we wished to investigate whether Stat3 itself may be a target for inhibition via phosphorylation on serine 727 by the p38 pathway. This was addressed directly using a Stat3 S727A mutant that abolishes serine phosphorylation at this site (58). CA-MKK3 and CA-MKK6 inhibited tyrosine phosphorylation of Stat3 S727A after IL-6 treatment (Fig. 9*A*, *top panel*). These results indicate that phosphorylation of Stat3 on serine 727 cannot explain the inhibitory effects seen in our experiments

To determine whether inhibition occurred upstream of Stat3 in the IL-6 signaling pathway, the levels and activation of the Jak1 kinase, which is the principal Jak required for IL-6 action (62, 63), were analyzed. Treatment of U266 cells (in which IL-6 strongly activates the Jak-Stat pathway, therefore allowing activation of

strong, as expected. As with IL-6, CA-MKK3 and CA-MKK6 effectively blocked this induction, but inhibition mediated by CA-MEK1 was minimal. In contrast, comparable inhibition by CA-MKK3, CA-MKK6, and CA-MEK1 was seen when LIF, which

Activation of p38 did not result in inhibition of Jak1 or Jak2 when these kinases were expressed in transfected cells, suggesting that Jaks are not direct targets of p38 and are not inhibited when

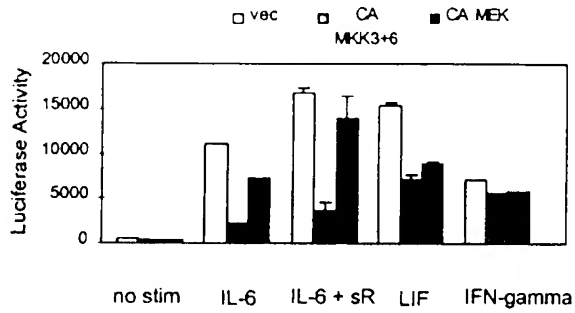


FIGURE 8. Constitutively active MKK3 and MKK6 inhibit reporter gene activity in response to IL-6-related cytokines. 293T cells were transfected as described in Fig. 6, except that a 4× IRF IFN-γ-activated sequence-luciferase reporter gene was included in the transfections. After 24 h cells were split onto replicate plates and subsequently stimulated with cytokines (50 ng/ml) for 3 h before assaying for luciferase activity. Luciferase activity was normalized relative to β-galactosidase activity encoded by a cotransfected internal control plasmid. One representative experiment of three is shown. SR, Soluble ligand-binding α subunit of IL-6R.

6→p38 pathway, we studied signaling through a truncated fusion receptor that consists of the extracellular domain of TrkC (binds neurotropin-3 (NT3)) and the membrane-proximal 113 aa of the gp130 cytoplasmic domain (of 273 aa present in the wild-type gp130 cytoplasmic domain) to which a Stat3 docking site (GGYMPQ) is fused (64). This receptor signals and activates Stat3 when addition of NT3 leads to dimerization (64). This receptor lacks the Y759 SH2 domain-containing protein-tyrosine phosphatase/SOCS3 docking site (55, 56, 64), therefore allowing inhibition to be studied in the absence of potential inhibitory interactions between gp130 and such molecules. 293T cells were transiently transfected with vector alone, CA-MKK3 and CA-MKK6, or CA-MEK1, and FLAG-tagged Stat3. NT3-induced signaling through the truncated receptor (TrkC-gp130-TGtY$_5$), as assayed by Stat3 DNA binding and tyrosine phosphorylation, was inhibited by CA-MKK3 and CA-MKK6 (Fig. 9*C*, *first* and *second panels*, *lane 4* vs *2*) in a fashion similar to the inhibition seen when signaling was triggered via the wild-type IL-6 receptor (Fig. 7). These results indicate that the target of the MKK3/6→p38 inhibitory pathway is different from Y759, which is the target of SOCS3-mediated inhibition. Interestingly, CA-MEK1 was unable to inhibit Stat3 activation in this system (Fig. 9*C*), even though it strongly activated the ERKs (data not shown), consistent with results reported by Terstegen et al. showing that ERK-mediated inhibition of gp130 targets Y759 (65).

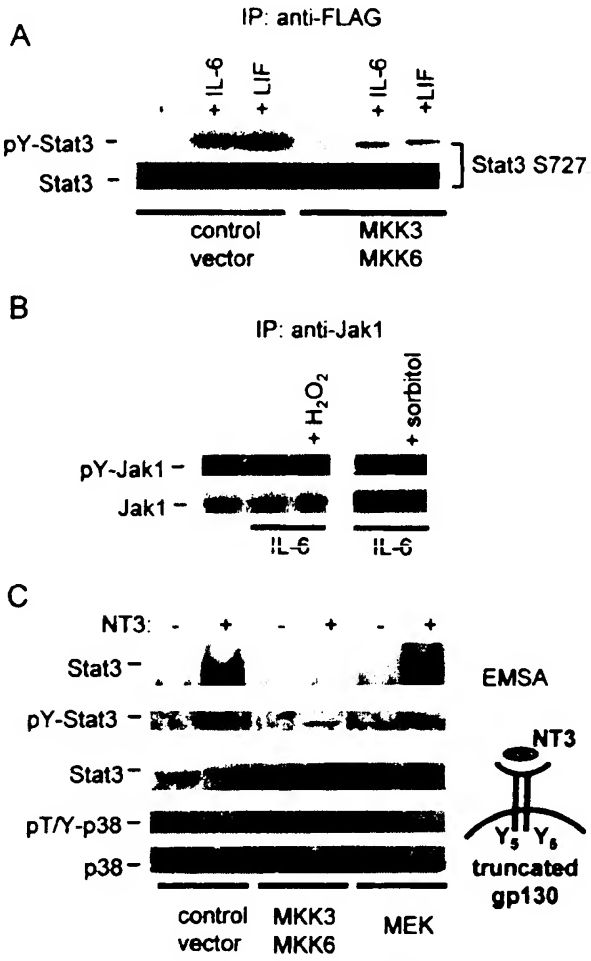


FIGURE 9. Inhibition of IL-6 signaling by MKKs/p38 occurs upstream of Stat3 activation. *A*, 293T cells were transfected as in Fig. 6, except the Stat3 S727A mutant was used. *B*, U266 cells were treated with H_2O_2 or sorbitol for 20 min before a 12-min stimulation with IL-6. Jak1 immunoprecipitates were analyzed using immunoblotting with a phosphotyrosine-specific Ab (4G10) and subsequently with a Jak1 mAb. *C*, 293T cells were transfected with a chimeric receptor construct (TrkC-gp130-TGtY$_5$) (64) encoding the ligand binding domain (LBD) of the TrkC receptor and a truncated form of gp130, bearing a single tyrosine for Stat3 docking. Cells were cotransfected with a control vector or CA kinases as described above and were treated with NT3 (100 ng/ml) for 12 min.

Discussion

We have investigated the molecular basis for interactions between IL-1, an inflammatory cytokine, and IL-6, a pleiotropic cytokine. IL-1 and IL-6 regulate each other's expression and are often expressed together at sites of inflammation (2, 3). IL-1 inhibited IL-

Jak-Stat signaling involve synthesis of inhibitory proteins such as SOCS (18–20, 54), potentially target the Stat molecule itself on a conserved carboxyl-terminal serine (serine 727 in Stat3) (58, 60),

SOCS, independent of modification of Stat3 on serine 727, and occurs at least in part upstream of Stat3 in the IL-6 signaling pathway. p38 is activated by stimuli distinct from those that activate ERK (30) and appears to target the membrane-proximal region of gp130, which is different from the molecular target of inhibition mediated by ERKs (37, 65) (Figs. 8 and 9). Thus, these findings identify a novel mechanism of inhibition of cytokine Jak-Stat signaling and have important implications for regulation of the hal-

clude a strong correlation between activation of the p38 kinase by stress agents (IL-1, sorbitol, UV, and H_2O_2) and inhibition of IL-6 activation of Stat3 (Table I), and reversal of inhibition by the p38

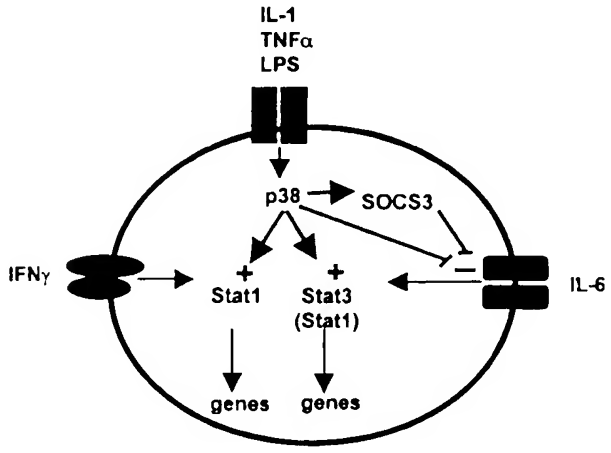
by the MEK/ERK inhibitor PD98059. Most convincingly, overexpression of constitutively active MKK3 and MKK6 kinases, which are immediately upstream of p38, served to block Stat3 activation in response to IL-6, resulting in inhibition of DNA binding, tyrosine phosphorylation, and Stat-dependent reporter gene activity (Figs. 7 and 8). The constitutively active MKKs did not activate either the ERKs or the JNKs in our system, confirming that activation of p38 alone was sufficient to inhibit Stat3 activation. It remains possible that the most downstream effector molecule in the MKK3/6→p38 pathway is not p38 but a substrate, possibly a kinase such as MAPK-activated protein (MAPKAP) kinase-2, and we have not excluded that JNKs may play a role in inhibition of IL-6 when cells are exposed to stimuli that activate both p38 and JNKs.

Recent reports show that inflammatory agents such as TNF-α and LPS activate SOCS3 expression, probably via the p38 pathway (51), and SOCS3 docks onto gp130 and inhibits signaling by IL-6 (55, 56). These results have led to the suggestion that TNF and LPS inhibit IL-6 signaling by a SOCS3-dependent mechanism, although to date the evidence for this is correlative. The mechanism of inhibition described in this report is different, in that inhibition is independent of de novo induction of genes, such as SOCS (Fig. 5), occurs in B cells when SOCS3 expression is not induced (Figs. 2*B* and 4*B*), and occurs when the SOCS3 docking site is deleted from gp130 (Fig. 9). Thus, inhibition of IL-6 can occur by two different p38-dependent pathways (see Fig. 10). Since levels of SOCS3 can remain elevated 4–12 h after addition of LPS to macrophages (50), one may therefore postulate that at early time points after addition of IL-1 or LPS, both the direct p38-dependent and the indirect p38- and SOCS3-dependent pathways may function in inhibition of Stats, whereas at later time points the SOCS3 pathway may predominate. The delayed, possibly SOCS3-dependent, pathway also inhibits IFN-γ signaling (50) and thus is less specific in terms of cytokines that are inhibited than the direct p38-dependent inhibitory pathway described herein. In addition, the relative importance of these pathways may be cell type specific, depending on the intensity and duration of MAPK and SOCS induction. Our data indicate that at early time points, the SOCS3-independent inhibitory pathway is necessary for inhibition of IL-6 signaling to occur.

When cells are treated with high concentrations of sorbitol, stress kinases can contribute to Stat activation (68, 69). Sorbitol (600 mM) was required for effective Stat activation, which was independent of gp130 or Jaks, but seemed to depend upon cell shrinkage (68, 69). We did not detect Stat activation when 400 mM sorbitol was used, possibly secondary to low levels of Stat activation at this concentration, as previously reported (68, 69). Our results are consistent with the experience from a large number of laboratories that physiologic activators of stress kinases, such as IL-1 and TNF, do not typically activate Stat tyrosine phosphorylation or DNA binding (70).

An important issue is the identity of the molecular target(s) of p38-mediated inhibition. Previous work on the interactions between MAPK and Jak-Stat pathways has focused on MAPK-dependent phosphorylation of a conserved carboxyl-terminal serine residue in the Stat proteins themselves (46, 58–61, 66, 71–79). There is general agreement that phosphorylation of Stat1 and Stat3 on serine 727 enhances the transcriptional potency of tyrosine-phosphorylated Stat dimers (46, 61, 74, 76, 78, 79), and one study suggests that DNA binding is enhanced as well (72). However, several studies have suggested that serine phosphorylation of Stats actually suppresses tyrosine phosphorylation and DNA binding (58, 60, 66, 80), although only one of these studies tested this directly using a mutated Stat (58). We have not excluded that serine phosphorylation of Stats may contribute to modulating Stat activity in our system, but several lines of evidence suggest that the predominant site of inhibition occurs upstream of Stats. Inhibition of Jak1 (Fig. 9), the Jak most important for IL-6 signaling (62, 63), indicates that inflammatory/stress stimuli inhibited IL-6 signaling at least in part upstream of Stat activation. Additional support for inhibition upstream of Stats includes the following. 1) Inhibition correlated with the receptor, and not with the Stat, that was activated. Thus, Stat1 and Stat3 activation was blocked when IL-6 or IL-10 was used, but not when IFN-γ or IFNα was used (Fig. 1) (37). 2) Inhibition of Stat3 mutated at serine 727 (Fig. 9); it is unlikely that inhibition of Stat3-S727A can be explained on the basis of phosphorylation of other serine residues in Stat3, since phosphopeptide mapping experiments have shown that serine 727 is the predominant site of serine phosphorylation (46, 58, 72, 74). 3) Inhibition of IL-6 activation of ERKs (which are downstream of Jaks, but independent of Stats) occurred in primary fibroblasts, where, in contrast to hemopoietic cells, IL-6 activation of ERKs was detectable (L. Ivashkiv, unpublished observations).

The two most likely targets of inhibition upstream of Stat3 in the IL-6 signaling pathway are the Jaks or the IL-6R. Although Jak2 can be phosphorylated on serine/threonine residues and inhibited by PKCδ (67), Jaks were not inhibited by p38 in cotransfection experiments (S. Ahmed, unpublished observations). Additional ev-



...signaling via JAK-independent p38-mediated pathways: a direct pathway and one that is SOCS3 dependent. The direct pathway is necessary for inhibition to occur at early time points after addition of IL-1, TNF, or LPS. The SOCS3 pathway may be the predominant pathway at later time points.

...much stronger activator of Jak1 than IL-6 in the cells that were used (38) (L. Ivashkiv, unpublished observations), the specificity of inhibition observed in Fig. 1 may represent a quantitative, rather

the gp130 cytoplasmic tail, which contains one consensus phosphorylation site for MAPKs, and a serine-rich region (1, 64). Mutation of the MAPK site resulted in an inactive signaling receptor (S. Ahmed, unpublished observations), probably because this site overlaps Jak-receptor interaction sites, and the effects of mutations in the serine-rich region will be tested in future experiments. Another key feature of the truncated receptor is that it lacks the Y759 SH2 domain-containing protein-tyrosine phosphatase/SOCS3 docking site (55, 56, 64), lending further support for the SOCS-independent nature of the inhibition seen in our system. Interestingly, the MEK→ERK pathway failed to inhibit signaling through the truncated gp130 fusion receptor, demonstrating a difference between the p38- and ERK-mediated pathways of inhibition. This is consistent with the observation that ERK-mediated inhibition of IL-6 signaling is dependent on the SOCS3-gp130 interaction (65) via the Y759 docking site. Therefore, different cytoplasmic sequences of the IL-6R complex may be targets for different MAPK pathways.

The opposing actions of IL-1 and MAPK pathways on the IL-6R (inhibit signaling) and on Stat1 and Stat3 (activate transcriptional potency of Stats that are tyrosine phosphorylated and dimerized) may appear paradoxical, but it is becoming increasingly clear that many cytokines and growth factors simultaneously activate multiple signals that may act synergistically or oppose each other (Fig. 10). Regulation of the balance between positive and negative signals also provides an opportunity for cells to fine-tune signals and often determines the ultimate action of cytokines (27, 81). As such, p38 may have both a positive and a negative regulatory role in IL-6 signaling. Inhibition upstream of Stats would result in a lower nuclear concentration of Stat dimers, but these Stats would be serine phosphorylated and transcriptionally active (Fig. 10). Under these conditions, there would be continuing high level expression of promoters that compete effectively for Stats when nuclear Stats are present in limiting amounts, but extinction of expression of genes whose promoters no longer bind Stats. This represents a plausible model to explain the complex effects of IL-1 on IL-6 signaling, in which some IL-6-inducible genes are superactivated, but others are suppressed (14, 32, 33, 35). In contrast to the situation with IL-6, IFN-γ signaling was not blocked by inflammatory cytokines when these cytokines were added 20 min before adding IFN-γ (Fig. 1), but Stat1 is serine phosphorylated and transcriptionally activated by p38 (77, 79). This is predicted to result in increased expression of IFN-γ-inducible genes, as previously reported (50, 71).

Blocking of the anti-inflammatory actions of IL-6 and IL-10 may be important to allow an inflammatory reaction to proceed in the face of expression of the counter-regulatory factors that are often highly expressed at sites of inflammation (2, 31). Interestingly, IL-6 activated expression of three genes, PIAS1, PIAS3, and the PGE_2 receptor, that probably subserve inhibitory or anti-inflammatory functions (44, 52), and IL-1 suppressed IL-6 induction of these genes (Fig. 4A). However, consistent with the complex interplay between IL-1 and IL-6, several patterns of gene regulation were seen, including activation of genes by both IL-1 and IL-6 (SOCS3), activation by IL-6 but not IL-1 (SOCS1), and suppression by IL-1 but not IL-6 (PIAS3). This is consistent with the observation that IL-1 and IL-6 activate overlapping but distinct sets of genes in hepatocytes (66), and will be further investigated by demonstrating in future work examining larger arrays of genes. Stress inhibition of IL-6 signaling also suppressed the expression of Stat-dependent IRF-1, Stat1, SOCS1 and PIAS3 genes in U266 cells (data not shown). These results therefore indicate that inhibition of Stat3 activation by inflammatory/stress factors has important functional consequences for the regulation of cell physiology.

In conjunction with our previous results (37), we have now demonstrated that both the ERK and p38 pathways are capable of inhibiting Jak-Stat signaling by differing mechanisms. A large number of receptors important in immune function, including FcRs, complement receptors, Ag receptors, costimulatory molecules, and inflammatory cytokines, activate MAPKs, and FcRs (83), complement receptors (84), and the TCR (43) have been shown to inhibit cytokine Jak-Stat signaling. Thus, modulation of Jak-Stat signaling by MAPKs may play an important role in regulation of the immune cell phenotype.

Acknowledgments

We thank Selina Chen-Kiang, Keith Elkon, and Philip King for reviewing the manuscript. We are grateful to Jackie Bromberg and Leif Bergsagel for providing cells, to C. Beadling and K. Smith for providing oligonucleotides, and to Roger Davis, J. Woodgett, Neil Stahl, and George Yancopoulos for providing plasmids. We are grateful to David P. Deon and Wai Ping Li for their expert technical assistance.

References

1. Taga, T., and T. Kishimoto. 1997. Gp130 and the interleukin-6 family of cytokines. *Annu. Rev. Immunol. 15:797.*
2. Ivashkiv, L. B. 1996. Cytokine expression and cell activation in inflammatory arthritis. *Adv. Immunol. 63:337.*
3. Dinarello, C. A. 1998. Interleukin-1β, interleukin-18, and the interleukin-1β converting enzyme. *Ann. NY Acad. Sci. 856:1.*
4. Eugster, H. P., K. Frei, M. Kopf, H. Lassmann, and A. Fontana. 1998. IL-6-defici mice resist myelin oligodendrocyte glycoprotein-induced autoimmune ence; al myelitis. *Eur. J. Immunol. 28:2178.*
5. Roma.), M., M. Sironi, C. Toniatti, N. Polentarutti, P. Fruscella, P. Ghezzi, R. Faggioni, W. Luini, V. van Hinsbergh, S. Sozzani, et al. 1997. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity 6:315.*
6. Trepicchio, W. L., and A. J. Dorner. 1998. Interleukin-11: a gp130 cytokine. *Ann. NY Acad. Sci. 856:12.*
7. Leng, S. X., and J. A. Elias. 1997. Interleukin-11 inhibits macrophage interleukin-12 production. *J. Immunol. 159:2161.*
8. Oh, J. W., N. J. Van Wagoner, S. Rose-John, and E. N. Benveniste. 1998. Role of IL-6 and the soluble IL-6 receptor in inhibition of VCAM-1 gene expression. *J. Immunol. 161:4992.*
9. Gatsios, P., H. D. Haubeck, E. Van de Leur, W. Frisch, S. S. Apte, H. Greiling, P. C. Heinrich, and L. Graeve. 1996. Oncostatin M differentially regulates tissue inhibitors of metalloproteinases TIMP-1 and TIMP-3 gene expression in human synovial lining cells. *Eur. J. Biochem. 241:56.*
10. Richards, C. D., C. Langdon, F. Botelho, T. J. Brown, and A. Agro. 1996. Oncostatin M inhibits IL-1-induced expression of IL-8 and granulocyte-macrophage colony-stimulating factor by synovial and lung fibroblasts. *J. Immunol. 156:343*
11. Trepicchio, W. L., M. Bozza, G. Pedneault, and A. J. Dorner. 1996. Recombinant human IL-11 attenuates the inflammatory response through down-regulation of proinflammatory cytokine release and nitric oxide production. *J. Immunol. 157 3627.*
12. La Flamme, A. C., A. S. MacDonald, and E. J. Pearce. 2000. Role of IL-6 in directing the initial immune response to schistosome eggs. *J. Immunol. 164:2419.*
13. Trepicchio, W. L., L. Wang, M. Bozza, and A. J. Dorner. 1997. IL-11 regulates macrophage effector function through the inhibition of nuclear factor-κB. *J. Immunol. 159:5661*
14. Baumann, H., K. R. Prowse, S. Marinkovic, K. A. Won, and G. P. Jahreis. 1989. Stimulation of hepatic acute phase response by cytokines and glucocorticoids. *Ann. NY Acad. Sci. 557:280.*
15. Papanicolaou, D. A., R. L. Wilder, S. C. Manolagas, and G. P. Chrousos. 1998. The pathophysiologic roles of interleukin-6 in human disease. *Ann. Intern. Med. 128:127.*
16. [illegible] A. Matsumoto, S. Tanimura, M. Ohtsubo, H. Misawa, et al. 1997. A new protein containing an SH2 domain that inhibits JAK kinases. *Nature [illegible]*
17. Naka, T., M. Narazaki, M. Hirata, T. Matsumoto, S. Minamoto, A. Aono, N. Nishimoto, T. Kajita, T. Taga, K. Yoshizaki, et al. 1997. Structure and func[illegible]

21 Xing, Z., J. Gauldie, G. Cox, H. Baumann, M. Jordana, X. F. Lei, and M. K. Achong. 1998. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J. Clin. Invest. 101:311*

22 van de Loo, F. A., S. Kuiper, F. H. van Enckevort, O. J. Arntz, and W. B. van den Berg. 1997. Interleukin-6 reduces cartilage destruction during experimental arthritis: a study in interleukin-6-deficient mice. *Am. J. Pathol. 151:177.*

23. Walmsley, M., D. M. Butler, L. Marinova-Mutafchieva, and M. Feldmann. 1998. An anti-inflammatory role for interleukin-11 in established murine collagen-induced arthritis. *Immunology 95:31.*

24. Trepicchio, W. L., M. Ozawa, I. B. Walters, T. Kikuchi, P. Gilleaudeau, J. L. Bliss, U. Schwertschlag, A. J. Dorner, and J. G. Krueger. 1999. Interleukin-11 therapy selectively downregulates type I cytokine proinflammatory pathways in psoriasis lesions. *J. Clin. Invest. 104:1527.*

25. Darnell, J. E., Jr. 1997. STATs and gene regulation. *Science 277:1630.*

26. Takeda, K., T. Kaisho, N. Yoshida, J. Takeda, T. Kishimoto, and S. Akira. 1998. Stat3 activation is responsible for IL-6-dependent T cell proliferation through preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice. *J. Immunol. 161:4652.*

27. Ohtane, T., K. Ishihara, T. Atsumi, K. Nishida, Y. Kaneko, T. Miyata, S. Itoh, M. Narimatsu, H. Maeda, T. Fukada, et al. 2000. Dissection of signaling cascades through gp130 in vivo: reciprocal roles for STAT3- and SHP-2-mediated signals in immune responses. *Immunity 12:95.*

28. Takeda, K., B. E. Clausen, T. Kaisho, T. Tsujimura, N. Terada, I. Forster, and S. Akira. 1999. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity 10:39.*

29. Saklatvala, J., J. Dean, and A. Finch. 1999. Protein kinase cascades in intracellular signalling by interleukin-1 and tumour necrosis factor. *Biochem. Soc. Symp. 64:63.*

30. Karin, M. 1998. Mitogen-activated protein kinase cascades as regulators of stress responses. *Ann. NY Acad. Sci. 851:139.*

31. Feldmann, M., F. M. Brennan, and R. N. Maini. 1996. Role of cytokines in rheumatoid arthritis. *Annu. Rev. Immunol. 14:397.*

32. Ito, A., Y. Itoh, Y. Sasaguri, M. Morimatsu, and Y. Mori. 1992. Effects of interleukin-6 on the metabolism of connective tissue components in rheumatoid synovial fibroblasts. *Arthritis Rheum. 35:1197.*

33. Poli, G., A. L. Kinter, and A. S. Fauci. 1994. Interleukin 1 induces expression of the human immunodeficiency virus alone and in synergy with interleukin 6 in chronically infected U1 cells: inhibition of inductive effects by the interleukin 1 receptor antagonist. *Proc. Natl. Acad. Sci. USA 91:108.*

34. Shingu, M., T. Isayama, C. Yasutake, T. Naono, M. Nobunaga, K. Tomari, K. Horie, and Y. Goto. 1994. Role of oxygen radicals and IL-6 in IL-1-dependent cartilage matrix degradation. *Inflammation 18:613.*

35. Mackiewicz, A., T. Speroff, M. K. Ganapathi, and I. Kushner. 1991. Effects of cytokine combinations on acute phase protein production in two human hepatoma cell lines. *J. Immunol. 146:3032.*

36. Hernandez-Caselles, T., and O. Stutman. 1993. Immune functions of tumor necrosis factor. I. Tumor necrosis factor induces apoptosis of mouse thymocytes and can also stimulate or inhibit IL-6-induced proliferation depending on the concentration of mitogenic costimulation. *J. Immunol. 151:3999.*

37. Sengupta, T. K., E. S. Talbot, P. A. Scherle, and L. B. Ivashkiv. 1998. Rapid inhibition of interleukin-6 signaling and Stat3 activation mediated by mitogen-activated protein kinases. *Proc. Natl. Acad. Sci. USA 95:11107.*

38. Sengupta, T. K., E. M. Schmitt, and L. B. Ivashkiv. 1996. Inhibition of cytokines and JAK-STAT activation by distinct signaling pathways. *Proc. Natl. Acad. Sci. USA 93:9499.*

39. Castro, A., T. K. Sengupta, D. C. Ruiz, E. Yang, and L. B. Ivashkiv. 1999. IL-4 selectively inhibits IL-2-triggered Stat5 activation, but not proliferation, in human T cells. *J. Immunol. 162:1261*

40. Ziegler-Heitbrock, H. W., E. Thiel, A. Futterer, V. Herzog, A. Wirtz, and G. Riethmuller. 1988. Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. *Int. J. Cancer 41:456*

41. Sengupta, T. K., A. Chen, Z. Zhong, J. E. Darnell, Jr., and L. B. Ivashkiv. 1995. Activation of monocyte effector genes and STAT family transcription factors by inflammatory synovial fluid is independent of interferon γ. *J. Exp. Med. 181:1015.*

42. Schreiber, E., P. Matthias, M. M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with 'mini-extracts,' prepared from a small number of cells. *Nucleic Acids Res. 17:6419.*

43. Lee, I. H., W. P. Li, K. B. Hisert, and L. B. Ivashkiv. 1999. Inhibition of interleukin 2 signaling and signal transducer and activator of transcription (STAT)5 activation during T cell receptor-mediated feedback inhibition of T cell expan-

49. Finbloom, D. S., and K. D. Winestock. 1995. IL-10 induces the tyrosine phosphorylation of tyk2 and Jak1 and the differential assembly of STAT1α and STAT3 complexes in human T cells and monocytes. *J. Immunol. 155:1079.*

50. Stoiber, D., P. Kovarik, S. Cohney, J. A. Johnston, P. Steinlein, and T. Decker. 1999. Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine signaling 3 and suppresses signal transduction in response to the activating factor IFN-γ. *J. Immunol. 163:2640.*

51. Bode, J. G., A. Nimmesgern, J. Schmitz, F. Schaper, M. Schmitt, W. Frisch, D. Haussinger, P. C. Heinrich, and L. Graeve. 1999. LPS and TNFα induce SOCS3 mRNA and inhibit IL-6-induced activation of STAT3 in macrophages. *FEBS Lett. 463:365.*

52. Liu, B., J. Liao, X. Rao, S. A. Kushner, C. D. Chung, D. D. Chang, and K. Shuai. 1998. Inhibition of Stat1-mediated gene activation by PIAS1. *Proc. Natl. Acad. Sci. USA 95:10626.*

53. Starr, R., and D. J. Hilton. 1999. Negative regulation of the JAK/STAT pathway. *BioEssays 21:47.*

54. Yoshimura, A., T. Ohkubo, T. Kiguchi, N. A. Jenkins, D. J. Gilbert, N. G. Copeland, T. Hara, and A. Miyajima. 1995. A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. *EMBO J. 14:2816.*

55. Schmitz, J., M. Weissenbach, S. Haan, P. C. Heinrich, and F. Schaper. 2000. SOCS3 exerts its inhibitory function on interleukin-6 signal transduction through the SHP2 recruitment site of gp130. *J. Biol. Chem. 275:12848.*

56. Nicholson, S. E., D. De Souza, L. J. Fabri, J. Corbin, T. A. Willson, J. G. Zhang, A. Silva, M. Asimakis, A. Farley, A. D. Nash, et al. 2000. Suppressor of cytokine signaling-3 preferentially binds to the SHP-2-binding site on the shared cytokine receptor subunit gp130. *Proc. Natl. Acad. Sci. USA 97:6493.*

57. Schiemann, W. P., L. M. Graves, H. Baumann, K. K. Morella, D. P. Gearing, M. D. Nielsen, E. G. Krebs, and N. M. Nathanson. 1995. Phosphorylation of the human leukemia inhibitory factor (LIF) receptor by mitogen-activated protein kinase and the regulation of LIF receptor function by heterologous receptor activation. *Proc. Natl. Acad. Sci. USA 92:5361.*

58. Chung, J., E. Uchida, T. C. Grammer, and J. Blenis. 1997. STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. *Mol. Cell. Biol. 17:6508.*

59. Ng, J., and D. Cantrell. 1997. STAT3 is a serine kinase target in T lymphocytes: interleukin 2 and T cell antigen receptor signals converge upon serine 727. *J. Biol. Chem. 272:24542.*

60. Jain, N., T. Zhang, S. L. Fong, C. P. Lim, and X. Cao. 1998. Repression of Stat3 activity by activation of mitogen-activated protein kinase (MAPK). *Oncogene 17:3157.*

61. Turkson, J., T. Bowman, J. Adnane, Y. Zhang, J. Y. Djeu, M. Sekharam, D. A. Frank, L. B. Holzman, J. Wu, S. Sebti, et al. 1999. Requirement for Ras/Rac1-mediated p38 and c-Jun N-terminal kinase signaling in Stat3 transcriptional activity induced by the Src oncoprotein. *Mol. Cell. Biol. 19:7519.*

62. Rodig, S. J., M. A. Meraz, J. M. White, P. A. Lampe, J. K. Riley, C. D. Arthur, K. L. King, K. C. Sheehan, L. Yin, D. Pennica, et al. 1998. Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. *Cell 93:373.*

63. Guschin, D., N. Rogers, J. Briscoe, B. Witthuhn, D. Watling, F. Horn, S. Pellegrini, K. Yasukawa, P. Heinrich, G. R. Stark, et al. 1995. A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6. *EMBO J. 14:1421.*

64. Stahl, N., T. J. Farruggella, T. G. Boulton, Z. Zhong, J. E. Darnell, Jr., and G. D. Yancopoulos. 1995. Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science 267:1349.*

65. Terstegen, L., P. Gatsios, J. G. Bode, F. Schaper, P. C. Heinrich, and L. Graeve. 2000. The inhibition of interleukin-6-dependent STAT activation by mitogen-activated protein kinases depends on tyrosine 759 in the cytoplasmic tail of glycoprotein 130. *J. Biol. Chem. 275:18810*

66. Jain, N., T. Zhang, W. H. Kee, W. Li, and X. Cao. 1999. Protein kinase C delta associates with and phosphorylates Stat3 in an interleukin-6-dependent manner. *J. Biol. Chem. 274:24392*

67. Kovanen, P. E., I. Junttila, K. Takaluoma, P. Saharinen, L. Valmu, W. Li, and O. Silvennoinen. 2000. Regulation of jak2 tyrosine kinase by protein kinase C during macrophage differentiation of IL-3-dependent myeloid progenitor cells. *Blood 95:1626.*

68. Gatsios, P., L. Terstegen, F. Schliess, D. Haussinger, J. M. Kerr, P. C. Heinrich, and L. Graeve. 1998. Activation of the Janus kinase/signal transducer and activator of transcription pathway by osmotic shock. *J. Biol. Chem. 273:22962*

69. Bode, J. G., P. Gatsios, S. Ludwig, U. R. Rapp, D. Haussinger, P. C. Heinrich, and L. Graeve. 1999. The mitogen-activated protein (MAP) kinase p38 and its upstream activator MAP kinase kinase 6 are involved in the activation of signal

46. Wen, Z., Z. Zhong, and J. E. Darnell, Jr. 1995. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell 82:241*

47. Zhong, Z., Z. Wen, and J. E. Darnell, Jr. 1994. Stat3: a STAT family member

of STAT3 promotes GAF activation in macrophages. *Mol. Cell. Biol.*

72. Zhang, X., J. Blenis, H. C. Li, C. Schindler, and S. Chen-Kiang. 1995. Requirement of serine phosphorylation for formation of STAT promoter complexes. *S.*

74. Zhu, X., Z. Wen, L. Z. Xu, and J. E. Darnell, Jr. 1997. Stat1 serine phosphorylation occurs independently of tyrosine phosphorylation and requires an activated Jak2 kinase. *Mol. Cell. Biol. 17:6618.*
75. Gollob, J. A., C. P. Schnipper, E. A. Murphy, J. Ritz, and D. A. Frank. 1999. The functional synergy between IL-12 and IL-2 involves p38 mitogen-activated protein kinase and is associated with the augmentation of STAT serine phosphorylation. *J. Immunol. 162:4472.*
76. David, M., E. Petricoin III, C. Benjamin, R. Pine, M. J. Weber, and A. C. Larner. 1995. Requirement for MAP kinase (ERK2) activity in interferon α- and interferon β-stimulated gene expression through STAT proteins. *Science 269:1721.*
77. Goh, K. C., S. J. Haque, and B. R. Williams. 1999. p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons. *EMBO J. 18:5601.*
78. Kovarik, P., D. Stoiber, M. Novy, and T. Decker. 1998. Stat1 combines signals derived from IFN-γ and LPS receptors during macrophage activation. *EMBO J. 17:3660.*
79. Kovarik, P., D. Stoiber, P. A. Eyers, R. Menghini, A. Neininger, M. Gaestel, P. Cohen, and T. Decker. 1999. Stress-induced phosphorylation of STAT1 at Ser727 requires p38 mitogen-activated protein kinase whereas IFN-γ uses a different signaling pathway. *Proc. Natl. Acad. Sci. USA 96:13956.*
80. Woetmann, A., M. Nielsen, S. T. Christensen, J. Brockdorff, K. Kaltoft, A. M. Engel, S. Skov, C. Brender, C. Geisler, A. Svejgaard, et al. 1999. Inhibition of protein phosphatase 2A induces serine/threonine phosphorylation, subcellular redistribution, and functional inhibition of STAT3. *Proc. Natl. Acad. Sci. USA 96:10620.*
81. Pimentel-Muinos, F. X., and B. Seed. 1999. Regulated commitment of TNF receptor signaling: a molecular switch for death or activation. *Immunity 11:783.*
82. Catlett-Falcone, R., T. H. Landowski, M. M. Oshiro, J. Turkson, A. Levitzki, R. Savino, G. Ciliberto, L. Moscinski, J. L. Fernandez-Luna, G. Nunez, et al. 1999. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity 10:105.*
83. Feldman, G. M., E. J. Chuang, and D. S. Finbloom. 1995. IgG immune complexes inhibit IFN-γ-induced transcription of the FcγRI gene in human monocytes by preventing the tyrosine phosphorylation of the p91 (Stat1) transcription factor. *J. Immunol. 154:318.*
84. Marth, T., and B. L. Kelsall. 1997. Regulation of interleukin-12 by complement receptor 3 signaling. *J. Exp. Med. 185:1987.*

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 271, No. 8, Issue of February 23, pp. 4134–4137, 1996
Printed in U.S.A.

Preassociation of STAT1 with STAT2 and STAT3 in Separate Signalling Complexes Prior to Cytokine Stimulation*

(Received for publication, September 1, 1995, and in revised form, December 1, 1995)

Louis F. Stancato‡, Michael David§, Christin Carter-Su¶, Andrew C. Larner§, and William B. Pratt‡‖

From the Departments of ‡Pharmacology and ¶Physiology, University of Michigan Medical School, Ann Arbor, Michigan 48109 and the §Division of Cytokine Biology, Food and Drug Administration, Bethesda, Maryland 20892

A variety of cytokines and growth factors act through an induction of gene expression mediated by a family of latent transcription factors called STAT (signal transducers and activators of transcription) proteins. Ligand-induced tyrosine phosphorylation of the STATs promotes their homodimer and heterodimer formation and subsequent nuclear translocation. We demonstrate here that STAT protein heterocomplexes exist prior to cytokine treatment. When unstimulated HeLa cells are ruptured in hypotonic buffer without salt or detergent, immunoadsorption of either STAT1 or STAT2 from the resulting cytosol yields coimmunoadsorption of the other STAT protein. Similarly, STAT1-STAT3 heterocomplexes are coimmunoadsorbed from hypotonic cytosol. STAT1 and STAT2 or STAT1 and STAT3 translated in reticulocyte lysate spontaneously form heterocomplexes when the translation lysates are mixed at 0 °C. Our data suggest that interferon-α/β-induced tyrosine phosphorylation increases the stability of a preexisting, latent, STAT1-STAT2 signaling complex. Newly translated STAT1 binds in equilibrium fashion to STAT2 and STAT3, but we show that STAT2 and STAT3 exist in separate heterocomplexes with STAT1, consistent with a model in which STAT1 contains a common binding site for other STAT proteins.

It is now appreciated that a large number of cytokines, growth factors, and hormones act through a sequence of steps that includes binding to receptors in the cytokine receptor superfamily followed by activation of the JAK (Janus kinases) family of tyrosine kinases, which results in activation of STAT (signal transducers and activators of transcription) proteins that subsequently migrate to the nucleus where they initiate specific gene transcription (see Refs. 1 and 2 for review). The overall signaling pathway is utilized by the interferons (IFNs)[1] and a variety of other cytokines, including the interleukins, some colony stimulating factors (granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor), leukemia inhibitory factor (LIF), oncostatin M, and erythropoietin, as well as the peptide hormones prolactin and growth hormone. The JAK family of kinases includes JAK1, JAK2, JAK3, and Tyk2, and, to date, six STAT proteins have been identified.

One of the most studied cytokine signaling systems is interferon-mediated gene activation. Here it is known that interaction of IFN-α or IFN-β with the IFN-α/β receptor activates JAK1 and Tyk2 with resulting tyrosine phosphorylation of the 91-kDa STAT1 and the 113-kDa STAT2 proteins, which are then thought to form a STAT heterodimer that associates with a 48-kDa DNA-binding protein. This multiprotein unit is called the interferon-stimulated gene factor 3, and it appears to be the primary positive regulator of interferon-stimulated response element-controlled genes (3–5). Thus, the STAT proteins are essentially latent transcription factors residing outside of the nucleus (6), and *in vivo* activation is associated with interferon-stimulated gene factor 3 translocation to the nucleus (5, 7).

We started the work we report herein asking whether we could use the gentle techniques of cell rupture and immunoadsorption in hypotonic buffer that we have used to study steroid receptor and protein kinase heterocomplexes (8) to detect possible complexes between STAT proteins and hsp90. We were not able to detect any association of STAT1, -2, or -3 with hsp90, but we serendipitously made some fundamental observations regarding the formation of STAT protein complexes themselves. We found that rupture of HeLa cells in a hypotonic buffer followed by immunoadsorption of STAT1 yields coimmunoadsorption of STAT2 and *vice versa*. This suggests that in untreated cells the two proteins exist together in a complex but that the association is weak, as the complex is not observed after cell rupture in buffer containing 1% Triton X-100 and 150 mM NaCl. After stimulation with IFN-α or IFN-β, however, the STAT1-STAT2 complex survives the "harsh" rupture conditions with detergent and salt. After stimulation by IFN-γ, which is thought to induce gene expression through an interaction of tyrosine-phosphorylated STAT1 homodimers with γ activation sequences (9), no STAT1-STAT2 complex is seen under the harsh rupture conditions.

Our data point to a revision of the standard model where it has been assumed that signaling by IFN-α/β receptors leads to STAT protein phosphorylation with subsequent heterocomplex formation. Rather, tyrosine phosphorylation seems to increase the stability of a preexisting, latent signaling complex. In the absence of cytokine stimulation, we have found that STAT1 and STAT2 associate with each other in an equilibrium fashion

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to

Medical School, Ann Arbor, MI 48109-0632. Tel.: [illegible]; FAX: 313 763 4450.

[1] The abbreviations used are: IFN, interferon; LIF, leukemia inhibitory factor; hsp, heat shock protein; IFS, 2 + 2 hydroxy-1,1-bisphy[illegible]

Materials

[illegible]I-conjugated goat anti-mouse and anti-rabbit IgGs and [35]S-methionine (translation-grade) were obtained from DuPont NEN. Protein

A-Sepharose and goat anti-mouse and anti-rabbit IgG horseradish peroxidase conjugates were from Sigma. The rabbit antisera against STAT1 and STAT2 have been described previously (10). The rabbit antiserum against STAT3 was raised against a peptide identical to the 38 C-terminal amino acids of STAT3. The anti-STAT3 monoclonal IgG antibody and the monoclonal anti-phosphotyrosine antibody (4G10) were from Transduction Laboratories. Leukemia inhibitory factor was from Promega. Recombinant human IFN-α-2a was from Hoffman La-Roche, and recombinant human IFN-β was from Chiron Inc. (Emeryville, CA). Recombinant human IFN-γ was from Genentech. *In vitro* coupled transcription/translation kits were from Promega.

Methods

Cell Culture and Fractionation—HeLa cells and 3T3-F442A mouse fibroblasts were grown in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. Where indicated, HeLa cells were incubated with 1000 units/ml IFN-α or IFN-β, or 10 ng/ml of IFN-γ, for 20 min at 37 °C. 3T3-F442A fibroblasts were incubated with 25 ng/ml of LIF for 20 min at 37 °C. Cells were harvested by scraping into Earle's balanced saline followed by a second wash and centrifugation at 500 × *g*. The washed cells were suspended in 1 volume of HE buffer (10 mM Hepes, pH 7.35, 1 mM EDTA), which we call "hypotonic buffer," or in HE + 1% Triton X-100 + 150 mM sodium chloride ("harsh buffer") and ruptured by Dounce homogenization. Homogenates were then centrifuged for 10 min at 12,000 × *g* at 4 °C, with the supernatant from this step being the "cytosol" from which the STAT proteins were immunoadsorbed.

STAT Protein Immunoadsorption—STAT proteins were immunoadsorbed from 200-μl aliquots of cytosol by first incubating with 5 μl of antiserum for 1 h on ice, followed by incubation with 35 μl of a 20% slurry of protein A-Sepharose for 1 h on ice. Immunopellets were then washed 3 times with 1 ml of TEG buffer (10 mM TES, 4 mM EDTA, 10% glycerol, 50 mM NaCl, pH 7.6) at 4 °C.

Western Blotting of STAT Proteins—For assay of STAT-associated proteins, immune pellets were assayed by SDS-polyacrylamide gel electrophoresis and immunoblotting. SDS-polyacrylamide gel electrophoresis was performed in 7% slab gels as described previously (11). Immunoblotting was carried out by transferring proteins from acrylamide slab gels to Immobilon P transfer membranes, followed by incubation for 2 h at room temperature with 0.1% anti-STAT1 or anti-STAT2 rabbit antiserum or with 1 μg/ml anti-STAT3 monoclonal antibody. The immunoblots were then incubated a second time with the appropriate horseradish peroxidase-conjugated counterantibody to visualize the immunoreactive bands and a third time with the appropriate ^{125}I-labeled counter antibody for autoradiography. In most cases, with appropriate cutting of the immunoblot, STAT1, STAT2, and STAT3 can be assayed on a single immunotransfer.

Western Blotting of Tyrosine-phosphorylated STAT Proteins—For assay of tyrosine phosphorylation of STAT proteins, Immobilon P membranes previously probed for STAT proteins and subsequently visualized by immunostaining and autoradiography were first stripped of bound antibody by incubating the membrane for 30 min at 50 °C in stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol). The membranes were then incubated for 2 h at room temperature with 1 μg/ml anti-phosphotyrosine monoclonal antibody. The immunoblots were then incubated a second time with the appropriate horseradish peroxidase-conjugated counterantibody to visualize the immunoreactive bands.

In Vitro Transcription and Translation of STAT Proteins—STAT proteins were translated *in vitro* using the Promega TnT-coupled transcription/translation system in rabbit reticulocyte lysate, which allows for both transcription and translation of a protein to occur in a single step. All STAT proteins were translated using 1–3 μg of their respective plasmids (12–14). The translation reactions (50 μl) were carried out for 1 h at 30 °C. The samples were then placed on ice for 15 min to stop

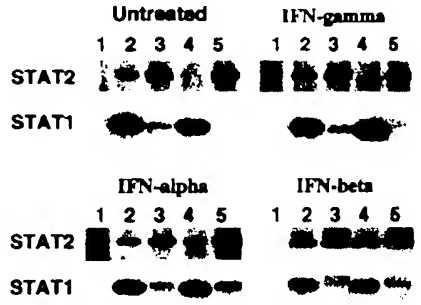


FIG. 1. **STAT1 and STAT2 are associated with each other prior to stimulation with IFN-α or IFN-β.** Untreated HeLa cells or HeLa cells treated with IFN-α, IFN-β, or IFN-γ were assayed for STAT protein complexes. Aliquots (200 μl) of cytosol prepared from hypotonic lysates (*lanes 1–3*) or from detergent/salt lysates (*lanes 4* and *5*) were immunoadsorbed with nonimmune rabbit serum, anti-STAT1, or anti-STAT2. After washing the immunopellets, STAT proteins were resolved by SDS-polyacrylamide gel electrophoresis and Western blotted with anti-STAT1 or anti-STAT2, as designated to the *left*. Under each condition of cell treatment, cytosols were immunoadsorbed with nonimmune serum (*lane 1*), anti-STAT1 (*lane 2*); anti-STAT2 (*lane 3*); anti-STAT1 (*lane 4*), and anti-STAT2 (*lane 5*).

the proteins are rather weakly bound (15) or 2) in the same buffer but with the addition of 1% Triton X-100 and 150 mM NaCl. This buffer contains detergent and salt to make it similar to the radioimmune precipitation assay buffer that is often used for cell rupture in studies of STAT protein phosphorylation (*e.g.* Ref. 16). It can be seen that immunoadsorption of hypotonic cytosols (from both untreated and interferon-treated cells) with antiserum to STAT1 yields coadsorption of STAT2 (*lane 2*) and that immunoadsorption of STAT2 yields coimmunoadsorption of some STAT1 (*lane 3*). Immunoadsorption of cytosols prepared from untreated or IFN-γ-treated cells with buffer containing detergent and salt does not result in coimmunoadsorption of the cognate STAT protein (*lanes 4* and *5*, *top row*). However, after treatment of cells with IFN-α or IFN-β, the heterocomplex survives cytosol preparation under harsh conditions (*lanes 4* and *5*, *bottom row*). By immunoblotting with anti-phosphotyrosine antibody, it was shown that STAT2 is phosphorylated after treatment with IFN-α and IFN-β but not after treatment with IFN-γ and that STAT1 was phosphorylated after treatment with all three IFNs (data not shown).

Because STAT1 and STAT2 were coimmunoadsorbed from hypotonic cytosols of untreated cells, we asked if the two proteins would spontaneously associate with each other when mixed together. STAT1 and STAT2 were transcribed and translated in rabbit reticulocyte lysate and then immunoadsorbed with anti STAT antisera. As shown in Fig. 2 (*lanes 7–9*), when the two proteins were translated in the same mix, immunoadsorption of either STAT1 or STAT2 yielded coimmunoadsorption of the other. It is unlikely that protein folding reactions are required to form the STAT protein heterocomplex because simply mixing two aliquots of reticulocyte lysate containing newly translated STAT1 or STAT2 at 0 °C yields a heterocomplex that can be immunoadsorbed (Fig. 2, *lanes 10*

[illegible] autoradiographs and Western blotting

RESULTS

STAT1 and STAT2 Are Preassociated—In the experiment of

[illegible] either hypotonic buffer or detergent containing buffer and the resulting cytosols were immunoadsorbed with anti STAT1 or anti STAT3 antisera. Immunoadsorption of hypotonic cytosol

FIG. 2. ***In vitro* translated STAT1 and STAT2 form a complex in rabbit reticulocyte lysate.** STAT1 and STAT2 were transcribed and translated in rabbit reticulocyte lysate. Shown is an autoradiogram of [^{35}S]methionine-labeled proteins in aliquots of reticulocyte lysate translation mixture or in immunoprecipitates prepared from the translation mixture. *Lane 1*, 5 μl of STAT1 translation mixture; *lane 2*, 5 μl of STAT2 translation mix; *lanes 3* and *4*, preimmune and anti-STAT1 immunoadsorption of STAT1 translation mix; *lanes 5* and *6*, nonimmune and anti-STAT2 immunoadsorption of STAT2 translation mix; *lanes 7–9*, nonimmune, anti-STAT1, or anti-STAT2 immunoadsorption from a translation mixture containing both STAT1 and STAT2 cDNAs; *lanes 10* and *11*, separate STAT1 and STAT2 translation mixtures combined on ice and then immunoadsorbed with nonimmune serum (*lane 10*) or anti-STAT1 antiserum (*lane 11*).

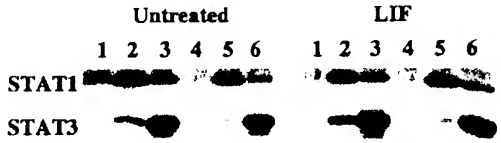


FIG. 3. **STAT1 and STAT3 exist in a preformed complex in 3T3-F442A cell cytosol.** Untreated or LIF-treated 3T3 cells were ruptured in hypotonic buffer (*lanes 1–3*) or buffer with detergent and salt (*lanes 4–6*), and the cytosols were immunoadsorbed with nonimmune serum (*lanes 1* and *4*), anti-STAT1 serum (*lanes 2* and *5*), or anti-STAT3 serum (*lanes 3* and *6*).

containing detergent and salt results in less coadsorption of the cognate STAT protein (*lanes 5* and *6*), and treatment with LIF does not increase heterocomplex recovery after salt treatment. In the experiment of Fig. 3, LIF stimulation of the 3T3 cells led to phosphorylation of both STAT1 and STAT3 as verified by immunoblotting with anti-phosphotyrosine antibody (data not shown).

As shown in Fig. 4 when STAT1 and STAT3 were cotranslated in the same translation mixture, immunoadsorption of STAT3 yielded coimmunoadsorption of STAT1 (*lanes 4* and *5*). Despite the fact that heterocomplexes could be demonstrated in a STAT1/STAT3 cotranslation mix as well as in a STAT1/STAT2 cotranslation mix (*lanes 6* and *7*), no heterocomplex could be demonstrated in STAT2/STAT3 cotranslation (*lanes 8* and *9*).

STAT2 and STAT3 Exist in Separate Heterocomplexes with STAT1—The data of Fig. 5*A* show that STAT2 and STAT3 are not recovered in the same heterocomplex with STAT1. 3T3 cells contain substantial amounts of all three STAT proteins, and it

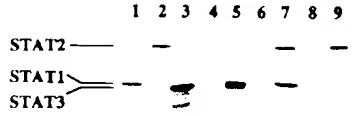


FIG. 4. **STAT1 and STAT3, but not STAT2 and STAT3, are coimmunoadsorbed after cotranslation in reticulocyte lysate.** Shown is an autoradiogram of [^{35}S]methionine-labeled proteins in aliquots of reticulocyte lysate translation mixture or immunoprecipitates prepared from the translation mixture. *Lanes 1–3*, 5 μl of STAT1, STAT2, and STAT3 translation mixtures, respectively; *lanes 4* and *5*, STAT1 and STAT3 cotranslated in the same aliquot of reticulocyte lysate, which was then immunoadsorbed with nonimmune serum or anti-STAT3 serum; *lanes 6* and *7*, STAT1 and STAT2 cotranslation immunoadsorbed with nonimmune or anti-STAT2 serum; *lanes 8* and *9*, STAT2 and STAT3 cotranslation immunoadsorbed with nonimmune or anti-STAT2 serum.

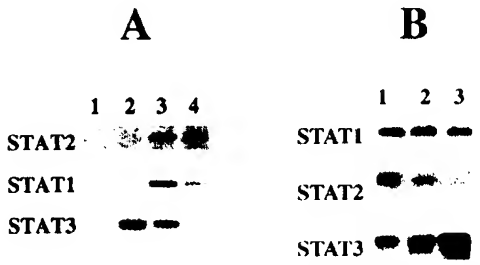


FIG. 5. **STAT1 is present in complexes with STAT2 or STAT3 but not both proteins simultaneously, and STAT2 and STAT3 compete for association with STAT1.** *A*, STAT2 and STAT3 are in separate complexes with STAT1. 3T3 cells were ruptured in hypotonic buffer and 200-μl aliquots of cytosol were immunoadsorbed with nonimmune serum (*lane 1*), anti-STAT3 (*lane 2*), anti-STAT1 (*lane 3*), or anti-STAT2 (*lane 4*), and immunoadsorbed proteins were detected by immunoblotting. *B*, STAT3 competes for STAT2 binding to STAT1. The three STATs were translated separately in reticulocyte lysate. STAT2 and STAT3 were then mixed in a ratio of 1:1 (*lane 1*), 1:2 (*lane 2*), or 1:10 (*lane 3*) prior to being mixed with a fixed amount of STAT1. The volumes of each reaction were equalized using untranslated reticulocyte extract. After 30 min on ice, the mixtures were immunoadsorbed with anti-STAT1 serum, and immunoadsorbed proteins were detected by immunoblotting.

shown in Fig. 5*B*, mixing of increasing amounts of STAT3 with STAT2 resulted in more STAT3 and less STAT2 being coimmunoadsorbed with STAT1 (*c.f. lanes 1* and *3*). As expected, the same amount of STAT1 was immunoadsorbed from each reaction.

The Y701F Mutant of STAT1 Forms a Heterocomplex with STAT2—It is known that phosphorylation of Tyr^{701} of STAT1 by JAK is necessary for interferon induced STAT1 nuclear translocation and gene activation (23). The experiment of Fig. 6 shows that the tyrosine at position 701 of STAT1 is not necessary for STAT1-STAT2 heterodimer formation. In this experiment, the Y701F mutant of STAT1 described by Shuai *et al.* (19) was cotranslated with STAT2. As shown in the figure, immunoadsorption of the cotranslation mixture with anti-STAT1 yields coadsorption of STAT2 (*lanes 5* and *10*), indicating normal heterodimer formation (c.f. *lane 10* with *lane 8*,

lane 4) yields coimmunoadsorption of only STAT1. These data suggest that binding of either STAT2 or STAT3 to STAT1 may prevent the binding of the other

of the developing basic model of signal transduction via STAT proteins (1, 2). The data presented in Fig. 1, however, are consistent with a variation of the model in which STAT1 and

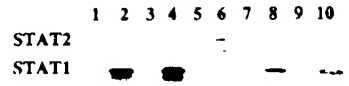


FIG. 6. **Mutation of Tyr^{701} of STAT1 to phenylalanine does not affect formation of the heterocomplex with STAT2.** Wild-type STAT1, the Y701F STAT1 mutant, and STAT2 were translated singly or in combination in reticulocyte lysate with [^{35}S]methionine, and the STAT proteins were immunoadsorbed. *Lanes 1* and *2*, wild-type STAT1 translation mix immunoadsorbed with nonimmune serum or anti-STAT1; *lanes 3* and *4*, Y701F STAT1 translation immunoadsorbed with nonimmune serum or anti-STAT1; *lanes 5* and *6*, STAT2 translation immunoadsorbed with nonimmune serum or anti-STAT2, *lanes 7* and *8*, wild-type STAT1 and STAT2 cotranslation immunoadsorbed with nonimmune serum or anti-STAT1; *lanes 9* and *10*, Y701F STAT1 and STAT2 cotranslation immunoadsorbed with nonimmune or anti-STAT1.

conditions of a radioimmune precipitation assay buffer. Phosphorylation of Tyr^{701} on STAT1 is required for its nuclear translocation (19), but phosphorylation does not seem to be required for STAT1 to form at least the low affinity complex with STAT2, as indicated by the observation that the Y701F mutant binds to STAT2 in reticulocyte lysate (Fig. 6). It seems likely that Tyr^{701} phosphorylation is required for the high affinity, detergent/salt-resistant STAT1-STAT2 complex demonstrated in Fig. 1.

STAT1 and STAT2 appear to bind to each other when mixed in solution (Fig. 2), as do STAT1 and STAT3 (Fig. 4). Although it is reduced, the STAT1-STAT3 complex is not eliminated under the harsh conditions of the detergent/salt buffer (Fig. 3), indicating that it is of higher affinity than the STAT1-STAT2 complex shown in Fig. 1. At this time, it is unclear whether STAT protein phosphorylation as a result of LIF treatment affects the affinity of the STAT1-STAT3 interaction. At least, under our experimental conditions, we have not observed a LIF effect on the salt sensitivity of the heterocomplex. It is interesting that immunoadsorption of STAT2 or STAT3 yields coimmunoadsorption of STAT1 but not each other (Fig. 5*A*). Although this could be explained by the location of epitopes for the immunoprecipitating antibodies in a STAT2/STAT3 interaction site, it is more likely that STAT2 and STAT3 form separate heterocomplexes with STAT1. As the binding of one of these STATs to STAT1 seems to preclude binding of the other, the binding sites may overlap, or there may be a common binding site on STAT1 for the other STAT proteins as suggested by the competition experiment of Fig. 5*B*.

The work we report here raises the notion that STAT protein heterocomplexes preexist in the cytoplasm. It also has been shown by coimmunoadsorption that JAK kinases are constitutively associated with several members of the cytokine receptor superfamily (20, 21), and it is possible that the STAT protein heterodimers are associated, albeit weakly, with this receptor-attached multiprotein structure. Such a notion is consistent with the fact that STATs can be activated *in vitro* using only plasma membrane-enriched fractions of a variety of cultured cells (22) and with recent evidence of Stahl *et al.* (23) that direct interactions exist between STATs and modular tyrosine-based motifs in a number of cytokine receptors. One could then conceive of signals being passed via the phosphorylation of entirely preassociated proteins. Whether the STAT proteins then move through the cytoplasm by diffusion or in association with a general protein movement system, as suggested for the steroid receptors (24), is unknown.

Acknowledgment—We thank Xin-Yuan Fu for kindly providing the Y701F mutation.

REFERENCES

1. Shuai, K. (1994) *Curr. Opin. Cell Biol.* **6**, 253–259
2. Ihle, J. N. (1994) *Trends Endocrinol. Metab.* **5**, 137–143
3. Levy, D., and Darnell, J. E. (1990) *New Biol.* **2**, 923–928
4. Friedman, R. L., and Stark, G. R. (1985) *Nature* **314**, 637–639
5. Dale, T. C., Imam, A. M. A., Kerr, I. M., and Stark, G. R. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 1203–1207
6. Fu, X-Y., Kessler, D. S., Veals, S. A., Levy, D. E., and Darnell, J. E. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 8555–8559
7. Levy, D. E., Kessler, D. S., Pine, R., and Darnell, J. E. (1989) *Genes & Dev.* **3**, 1362–1371
8. Stancato, L. F., Chow, Y-H., Hutchison, K. A., Perdew, G. H., Jove, R., and Pratt, W. B. (1993) *J. Biol. Chem.* **268**, 21711–21716
9. Shuai, K., Horvath, C. M., Huang, L. H. T., Qureshi, S. A., Cowburn, D., and Darnell, J. E., Jr. (1994) *Cell* **76**, 821–828
10. Larner, A. C., David, M., Feldman, G., Igarashi, K., Hackett, R. H., Webb, D. S. A., Sweitzer, S. M., Petricoin, E. F., and Finbloom, D. S. (1993) *Science* **261**, 1730–1736
11. Bresnick, E. H., Dalman, F. C., Sanchez, E. R., and Pratt, W. B. (1989) *J. Biol. Chem.* **264**, 4992–4997
12. Schindler, C., Fu, X-Y., Improta, T., Aebersold, R., and Darnell, J. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 7836–7839
13. Fu, X-Y., Schindler, C., Improta, T., Aebersold, R., and Darnell, J. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 7840–7843
14. Akira, S., Nishio, Y., Inoue, M., Wang, X-J., Wei, S., Matsusaka, T., Yoshida, K., Sudo, T., Naruto, M., and Kishimoto, T. (1994) *Cell* **77**, 63–71
15. Hutchison, K. A., Dittmar, K. D., and Pratt, W. B. (1994) *J. Biol. Chem.* **269**, 27894–27899
16. Fu, X-Y. (1992) *Cell* **70**, 323–335
17. Campbell, G. S., Meyer, D. J., Raz, R., Levy, D. E., Schwartz, J., and Carter-Su, C. (1995) *J. Biol. Chem.* **270**, 3974–3979
18. Raz, R., Durbin, J. E., and Levy, D. E. (1994) *J. Biol. Chem.* **269**, 24391–24395
19. Shuai, K., Stark, G. R., Kerr, I. M., and Darnell, J. E. (1993) *Science* **261**, 1744–1746
20. Campbell, G. S., Argetsinger, L. S., Ihle, J. N., Kelly, P. A., Rillema, J. A., and Carter-Su, C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 5232–5236
21. Stahl, N., Boulton, T. G., Farruggella, T., Ip, N. Y., Davis, S., Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Barbieri, G., Pellegrini, S., Ihle, J. N., Yancopoulos, G. D. (1994) *Science* **263**, 92–95
22. David, M., and Larner, A. C. (1992) *Science* **257**, 813–815
23. Stahl, N., Farruggella, T. J., Boulton, T. G., Zhong, Z., Darnell, J. E., and Yancopoulos, G. D. (1995) *Science* **267**, 1349–1353
24. Pratt, W. B. (1993) *J. Biol. Chem.* **268**, 21455–21458